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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
Adenosine is a potential novel factor in breast cancer progression and this project has been designed to evaluate adenosine tumor-promoting activity experimentally. The initial period (12 months, Aug 1 01 to Jul 31 02) has been used to generate and characterize breast cancer cell lines that differ in adenosine removing and generating potency. We have accomplished these goals successfully and altered invasive and metastatic MDA-MD-231 cells that have a high rate of endogenous adenosine generation. Using retroviral gene transfer system we generated variants that: a) overexpress adenosine deaminase (ADA), an enzyme that removes endogenous adenosine, b) exhibit decreased expression of ecto-5'-nucleotidase (eN)with anti-sense cDNA, and c) overexpress EGFP, a control protein. All these cells have been cloned by limited dilution and their altered adenosine metabolism has been analyzed, and confirmed, in vitro. Furthermore, we used MCF-7 cells that are non-tumorigenic and do not express endogenous eN, to develop eN-expressing and EGFP-expressing variants. We have obtained control EGFP cells and are currently selecting for the eN-expressing clones. Thus, we are in position to initiate the second phase of the project and test selected cells for their angiogenic and tumorigenic potential in nude mice.

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Table of Contents

1. Introduction

Adenosine accumulates in solid tumors at high concentrations and has been shown to stimulate tumor growth and angiogenesis, inhibit cytokine synthesis, inhibit adhesion of immune cells to endothelial wall and inhibit the function of T-cells, macrophages and natural killer cells (4). However, the mechanisms whereby adenosine accumulates in cancer and the specific effects of adenosine *in vivo* are not well understood. This project has been designed to test the possibility that increased, or decreased extracellular adenosine concentrations in breast tumors, may promote or inhibit tumor grafts growth in nude mice, respectively.

Local concentrations of adenosine are regulated by the ratios of the adenosine-producing activity of ecto-5'-nucleotidase (ecto-5'-NT, eN) and the adenosine-removing activities of adenosine deaminase (ADA) and adenosine kinase (AK) (1-3). Our previous results indicate that expression of these enzymes is regulated in a coordinate manner, bringing about adenosine-removing or adenosine-generating phenotypes depending on the cell type and stimulus (5). Recently, we have discovered that the wnt oncogene, that is frequently found disregulated in human cancers, including breast carcinoma, induces overexpression of ecto-5'-NT and decreases the expression of ADA, thus providing a novel mechanism for the increased concentrations of extracellular adenosine during cancer progression (6). Furthermore, our more recent data show that estradiol also regulates both eN and ADA expression in a reciprocal way suggesting yet another mechanism for increasing adenosine concentrations during progression to ER- breast carcinoma (7).

These data show that during breast cancer progression, whether associated with altered wnt signaling or with the loss of ER, there is coordinate shift in expression of eN and ADA that results in dramatically increased generation of extracellular adenosine. Knowing the immunosuppressive, angiogenic and growth-promoting activities of adenosine, it is tempting to speculate that increased expression of eN and generation of adenosine in breast cancer is one of the mechanism to increase the invasive and metastatic behavior. As such, it may significantly contribute to the more aggressive course of the disease. Thus, if confirmed, adenosine generation in cancer may become a new important target for pharmacological intervention.

2. Body of the Report

In order to effectively manipulate graft adenosine generation and concentrations we have set to differentially express two major enzymes that either increase adenosine generation (ecto-5'-nucleotidase, eN) or decrease local adenosine (adenosine deaminase). As argued in our grant application, we have chosen two breast cancer cell lines that differ in tumorigenic and metastatic potential, MCF-7 that do not express eN and are poorly tumorigenic and MDA-MB-231, that express high eN and are highly tumorigenic in nude mice. We will be testing whether increased expression of eN in MCF-7 cell may increase tumorigenicity in xenograft model. We will also test if expression of ADA in MCF-7 cells will decrease their tumorigenicity when co-injected with Matrigel or estrogen pellets. Conversely, by decreasing of expression of eN (with anti-sense cDNA) in MDA-MB-231 we will analyze for potential decrease in tumorigenicity of these cells in nude mice. However, since MCF-7 cells are already weakly tumorigenic and expression of high ADA may not cause much change, we are also expressing ADA in MDA-MB-231 cells to counteract their high rate of adenosine generation and subsequently test these cells for their tumorigenicity. Toward this end, and to fulfill the first 18 months objectives of this project, we have produced several gene constructs and engineered a number of breast cancer cell lines.

The following minor changes were made in the research plan: I) instead of expression of control LacZ proteins we used EGFP, which expression in cells and grafts could be monitored non-invasively. II) as an additional control we have expressed mutated ADA that is enzymatically dead and therefore may serve as an ideal control for w/t ADA. These alterations do not in any way change the research plan of this proposal, rather, they enhance approved protocol and will strengthen the conclusions emanating from this project.

A. Cloning of ADA, eN and EGFP cDNA's.

We have chosen w/t sense and anti-sense eN, w/t ADA, mutated ADA (producing inactive protein) and EGFP (producing control unrelated protein). Mutated ADA, containing an inactivating mutation at the catalytic site, will serve as an excellent control for ADA, and EGFP will serve as a general control for unrelated protein. To achieve the high level of gene expression in target cells we used retroviral vector pLPCX (pur) and pLPCX2 (neo) (Clontech). In both cases expression is driven by CMV promoter. Target genes were amplified using primers with cloned-in appropriate restriction sites and were subcloned into pCR-2.1 vector from Invitrogen and sequenced to verify the absence of potential mutations generated during PCR. To reduce the likelihood of modifications, amplifications were performed with the high fidelity pfu Polimerase from Stratagene. Subsequent confirmed clones were cut out with Not I and Xho I restriction enzymes and subcloned into retroviral vectors pLPCX (pur) and pLPCX2 (neo). Standard protocols were used for ligation, transfection into E.coli strain and propagation. Specific clones were confirmed by restriction digests and one of each

constructs were grown in 0.5 liter of bacteria culture and purified for the purpose of transfection into mammalian cells using Maxi prep (Quagen).

B. Generation of viral particles for transfection.

PT-67 (Clontech) packaging cells were used to transfect with pLPCX2 plasmids harboring described cDNA's by either calcium phosphate or Fugene6 liposome-based transfection reagent (Roche). Transfection efficiency was tested with LacZ pCDNA3 plasmid. Cells were selected with the appropriate selection agent and supernantants containing viral particles were collected and stored at 4°C

C. Target cell infections and selection of overexpressing clones.

These were used to infect either MCF-7 or MDA-MB-231 cells in 3 to 4 subsequent rounds of exposure, 6 – 10 hours each, in a 6-well plates. After infection increasing concentration of G418, starting at 0.3 mg/ml, were applied every second day until non-infected cells in a control well died out. Polyclonal cell populations were then expanded and used to obtain clonal populations by limiting dilution in 96-well plates. Colonies originated from single cells were expanded and used to verify the expression levels of target proteins by Western blot (Figs 1-5) and enzyme activities of ADA and ecto-5'-nucleotidase (Table 1). Data show that we were able to obtain 20 to 30-fold increase in ADA expression in MCF-7 cells and 55 to 72-fold increase in MDA-MB-231 (Table 1). This level of expression is within the target activity of ADA set for this proposal. Using the anti-sense cDNA we were able to obtain MDA-MB-231 clones with up to 92% reduced endogenous eN. Clonal populations of the mutated ADA containing MCF-7 and MDA-MB-231 cells are being currently expanded and were not yet available for Western blot analysis and enzyme activity. All other selected clones are being currently tested for Mycoplasma contamination before using them for orthotopic inoculations in immunocompromized mice.

One major cell type that is still being currently developed is the MCF-7 cells overexpressing w/t ecto-5'-nucleotidase. Initial attempt failed to produce viable cell colonies and we are in a process of re-checking the construct sequence before repeating the target cell infection procedure. Also, we need to estimate the rates of AMP catabolism and adenosine generation in cell culture in developed cells. However, we still have remaining 6 of the 18 months of the initial period to accomplish these mid-term goals.

Personnel involved in this project:

Jozef Spychala, Ph.D., P.I.
Anna Ostapkowicz, Tech. II
Jonathan Kahler, temporary technician (hired to make up for delay caused by administrative procedures in hiring Tech II)

2. Key research accomplishments

We have developed and partially characterized following cell lines:

- MCF-7 cells with w/t ADA,
- MCF-7 cells with mutated ADA,
- MCF-7 cells with EGFP,
- MDA-MB-231 cells with ADA,
- MDA-MB-231 cells with mutated ADA,
- MDA-MB-231 cells with decreased eN (anti-sense cDNA),
- MDA-MB-231 cells with EGFP,

3. Reportable outcomes

Several new breast cancer cell sublines have been developed to study the effect of altered adenosine metabolism on graft growth in nude mice. These cells may now become available to other investigators in breast cancer research. In addition, these cells may also be valuable for other purposes where studying the effects of local adenosine and its signaling properties is relevant.

We report the findings that led to this project in an abstract form at the Era of Hope Meeting in Orlando in September 2002 (Appendices).

5. Conclusions

The initial 12-months research period has been devoted to develop tools that will be subsequently used to address the function of adenosine in breast cancer. These tools were successfully developed and are now available for further investigation. However, no conclusions as to the role of adenosine can yet be drawn.

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   In press

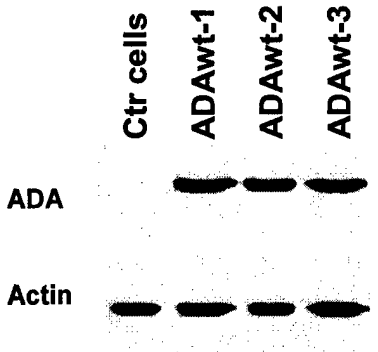

Fig. 1. Western blot of expressed w/t ADA in MCF-7 cells. Cell extract (30 ug) was loaded into each lane and probed with αADA antibodies (Santa Cruz, sc-7450)

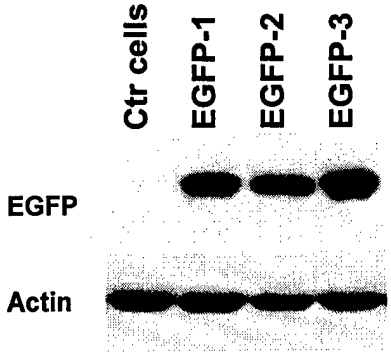

Fig. 2. Western blot of expressed EGFP in MCF-7 cells. Cell extract (30 ug) was loaded into each lane and blot probed with Living Colors Antibody (Clontech).

Fig. 3. Western blot of expressed w/t ADA in MDA-MB-231 cells. Experimental conditions as in Fig. 1.

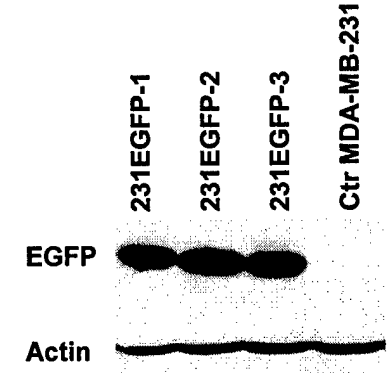

Fig. 4. Western blot of EGFP expressed in MDA-MB-231 cells. Experimental conditions as In Fig. 2.

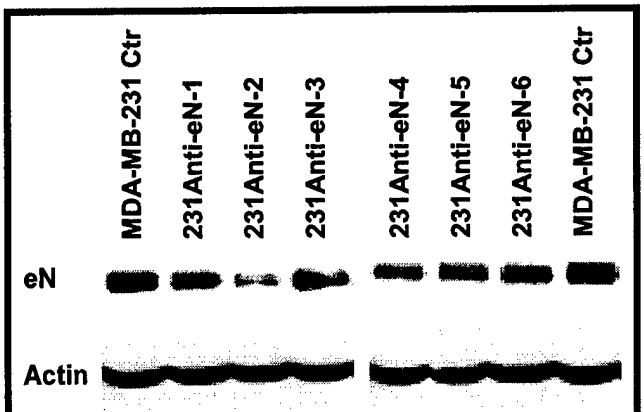

Fig. 5. Western blot of eN expression in eN anti-sense transfected MDA-MB-231 cells. Cell extract (30 ug) was loaded into each lane and probed with αeN antibodies (9).

TABLE 1. ADA and eN activities in stably transfected MCF-7 and MDA-MB-231 cells.

Cell line	ADA (nmoles/min/mg prot.)	eN (nmoles/min/mg prot.)
Values are averages, ± SD from 3 measurements		
MCF-7 Ctr	1.2 ± 0.3	
MCF-7ADAwt-1	26.1 ± 5.3	
MCF-7ADAwt-2	21.9 ± 4.4	
MCF-7ADAwt-3	31.2 ± 6.9	
MDA-MB-231 Ctr	0.4 ± 0.1	
231ADAwt-1	27.3 ± 6.7	
231ADAwt-2	22.0 ± 7.1	
231ADAwt-3	29.1 ± 5.9	
MDA-MB-231 Ctr		56.8 ± 12.1
231Anti-eN-1		21.7 ± 6.5
231Anti-eN-2		5.0 ± 1.5
231Anti-eN-4		10.1 ± 1.9

A SHIFT IN EXPRESSION OF COMPONENTS OF LIPID RAFTS IN ER NEGATIVE BREAST CANCER

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Several recent approaches using DNA arrays to screen for specific breast cancer subtypes revealed significant differences in gene expression profiles between ER positive and negative breast carcinoma. Since we have found dramatic overexpression of eN (ecto-5'-nucleotidase or CD73), a component of lipid rafts, in ER- breast cancer cell lines, we undertook investigation of the functional significance of eN in more aggressive and metastatic breast cancer cells. Toward this end, we have analyzed selective groups of membrane proteins as well as transcription factors that potentially may co-express with, or be involved in, the regulation of expression of eN. As a cellular model mimicking transition to ER-negative breast cancer we used the MCF-7 cell line and its two unrelated derivatives: drug resistant Adr2 and c-Jun transformed 2-33 clone. Our directed expression profiling revealed a remarkable coordinate shift in expression profiles of membrane proteins, such as MDR-1, caveolin-1, CD44, integrin beta 1 and signaling proteins, such as FAK, Lyn, Src, Lck, trimeric Gi-2. Among the cell surface antigens previously established as markers of breast crcinoma we have observed significant downeregulation of CD24 and uPAR. Since eN, CD24, uPAR and most of signaling molecules surveyed in this study are components of lipid rafts, the results from our limited profiling also suggest that there is a major remodeling of lipid rafts during transition to ER negative breast cancer. To investigate whether the change in lipid raft composition would have functional significance, we have clustered lipids rafts with lectin Concanavalin A and observed dose- and time-dependent increase in phosphorylation of c-Src and FAK in ER negative cells but not in MCF-7 cells. Furthermore, upon clustering of membrane glycoproteins we have also observed a significantly increased association of lipid rafts with cytoskeleton which was completely reversed by Latrunculin A, an inhibitor of actin polymerization. Thus, our results suggest that during transition to more aggressive breast cancer phenotype there is a significant structural re-organization of lipid rafts that leads to altered signaling properties of this membrane domain.

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